Micro-RNA Modulation of Insect Virus Replication

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Abstract

The outcome of virus infection in insects is impacted by regulation of both host and virus gene expression. A class of small RNAs called micro-RNAs (miRNA) have emerged as important regulators of gene expression that can influence the outcome of virus infection. miRNA regulation occurs at a comparatively late stage of gene expression, allowing for rapid control and fine-tuning of gene expression levels. Here we discuss the biogenesis of miRNAs from both host and virus genomes, the interactions that lead to regulation of gene expression, and the miRNA–mRNA interactions that lead to either antivirus or provirus consequences in the course of virus infection in insects.

Introduction: insect–virus interaction and microRNAs

The course and outcome of virus infection is reliant on intricate and complex host–virus interactions (Rapicetta *et al.*, 2002; Whitton *et al.*, 2005; Clyde *et al.*, 2006). With small genomes and limited coding capacity, viruses hijack host factors for almost every step of virus infection while simultaneously evading the host antiviral system (Ploegh, 1998; Ahlquist *et al.*, 2003; Cherry and Silverman, 2006). The virus exploits the host's cell factors and the host counters the viral assault by restricting viral access to cell factors and/or mounting its antiviral immune response (Ahlquist *et al.*, 2003, 2005; Cherry and Silverman, 2006; Huszar and Imler, 2008; Kemp and Imler, 2009; Delpeut *et al.*, 2012; Vodovar and Saleh, 2012; Xu and Cherry, 2014; Mussabekova *et al.*, 2017). Many molecular components mediate and are mediated by this host–virus cross-talk, including microRNAs.

microRNAs (miRNAs) are a large class of highly conserved, ≈22 nt non-coding RNAs that regulate gene expression. Compared to the more upstream regulatory mechanisms such as transcriptional regulation and chromatin remodelling, regulation by miRNA occurs at a later stage of gene expression, allowing for rapid control and fine-tuning of gene expression levels (Chen *et al.*, 2013). Complementary binding of at least the seed region (second to eighth nucleotide from the 5′-end) of the ≈22 nt long miRNA with target mRNAs influences mRNA stability and/or translational efficiency and consequently modulates genes involved in a spectrum of important cellular processes (Bartel, 2009; Fabian *et al.*, 2010; Pasquinelli, 2012), including immune response and host–virus interactions (Lindsay, 2008; Lodish *et al.*, 2008; Tsitsiou and Lindsay, 2009; Xiao and Rajewsky, 2009; O'Connell *et al.*, 2010; Li and Rana, 2014).

Changes in miRNA abundance during the host–virus interaction can direct the course of virus pathogenesis (Monsanto-Hearne and Johnson, 2018). There are two distinct motifs in terms of miRNA effect on virus infection: while regulation of some miRNAs have antivirus consequences, regulation of others have provirus consequences. Antivirus consequences can arise by either one of the two mechanisms: binding of host miRNA-host mRNA or host miRNA-virus mRNA. On the other

hand, provirus consequences can occur as a result of any of four binding events: host miRNA-host mRNA, host miRNA-virus mRNA, virus miRNA-virus mRNA, and finally virus miRNA-host mRNA. The growing body of literature on insect-virus infection in the context of miRNA regulation shows that there are many differentially regulated miRNAs that can have either antiviral or proviral outcomes. The net effect of cooperative and opposing impacts of the suite of miRNAs on the host–virus interaction then influences whether or not virus infection ensues. This chapter starts with a discussion on miRNA genomics, biogenesis, and regulation of target mRNAs, many of which have been established through the study of insect model *Drosophila melanogaster*. The chapter then details miRNA–mRNA interactions leading to either antivirus or provirus consequences that determine the course of virus infection in insects.

miRNA: genomics, biogenesis to regulation of target

miRNAs are ubiquitous in nature. Regarded as a mere curiosity upon the discovery of the first miRNA in *Caenorhabditis elegans* in 1993 (Bartel, 2018), miRNAs are now known to be encoded in the genome of a full spectrum of organisms, from unicellular flagellates (Zhao *et al.*, 2007) to multicellular plants and animals (Bartel and Chen, 2004; Bartel, 2009). At present, the more than 270 species recorded in the miRNA registry, miRBase, produce over 28,000 mature miRNAs. From these, around 5000 miRNAs have been documented for insects (Table 4.1) (Kozomara and Griffiths-Jones, 2014). The coverage of the insect miRNA groups is highly biased and incomplete (Ylla *et al.*, 2016), with more than a third of the insects and more than 40% of the currently known insect miRNAs in miRBase belonging to the genus *Drosophila* (Griffiths-Jones *et al.*, 2008; Kozomara and Griffiths-Jones, 2014). Viruses also encode miRNAs (Grundhoff and Sullivan, 2011; Kincaid and Sullivan, 2012), with over 500 mature miRNAs encoded in 34 virus genomes recorded (Griffiths-Jones *et al.*, 2008; Kozomara and Griffiths-Jones, 2014).

Host miRNA genomics

miRNAs are a widespread component of the insect genome. miRNAs account for an estimated 1–2% of genes of the model insect *D. melanogaster* (Lai *et al.*, 2003; Landgraf *et al.*, 2007; Lim *et al.*, 2003a,b; Ruby *et al.*, 2007), while the number of miRNAs in *Tribolium* is at least 15% larger than that of *Drosophila* (Marco *et al.*, 2010). miRNA

Table 4.1 Number of mature miRNAs produced by insect species in miRbase

Insect species	Number of mature miRNAs
Aedes aegypti	164
Anopheles gambiae	163
Apis mellifera	262
Acyrthosiphon pisum	97
Bactrocera dorsalis	78
Biston betularia	2
Bombyx mori	563
Culex quinquefasciatus	91
Dinoponera quadriceps	197
Drosophila ananassae	75
Drosophila erecta	120
Drosophila grimshawi	72
Drosophila melanogaster	469
Drosophila mojavensis	71
Drosophila persimilis	69
Drosophila pseudoobscura	271
Drosophila sechellia	120
Drosophila simulans	213
Drosophila virilis	328
Drosophila willistoni	72
Drosophila yakuba	103
Heliconius melpomene	97
Locusta migratoria	14
Manduca sexta	93
Nasonia giraulti	32
Nasonia longicornis	28
Nasonia vitripennis	53
Polistes canadensis	140
Plutella xylostella	127
Spodoptera frugiperda	221
Tribolium castaneum	590

loci are found throughout the genome: intergenic miRNAs are embedded between clusters of genes, while intronic miRNAs are found within introns of protein-coding regions. The former is transcribed independently by their own transcription units, whereas the latter are processed either by their host transcription units or by their own transcription units. Some miRNAs are encoded individually, while others are found in clusters with other miRNAs (Lee *et al.*, 2002; Kim and Kim, 2007; Carthew and Sontheimer, 2009).

miRNAs are transcribed from the genome using similar mechanisms as mRNAs. miRNA promoters are typically the same as mRNA-encoding promoters in terms of distance from the miRNA-encoding sequence and presence of recognition elements. The miRNA transcription initiation site can be as close as a few hundreds of base pairs (bp) to the miRNA-encoding sequence or can be as far as 20 kb. miRNA recognition promoter elements can include TATA box, transcription factor II B recognition element (BRE), initiator (Inr) motif 10 element, and downstream promoter element (Ozsolak *et al.*, 2008).

miRNA biogenesis to recognition of targets

Canonical biogenesis of host miRNA

miRNA biogenesis canonically begins with the transcription of miRNA-encoding genomic regions (Rodriguez *et al.*, 2004) by RNA polymerase II (Lee *et al.*, 2004a) (Fig. 4.1). The Pol II-generation of the miRNA primary transcripts (pri-miRNAs) is coupled with pri-miRNA processing. Similar to most other Pol II-generated products, pri-miRNAs are methylguanosine-capped at the 5′-end and polyadenylated at the 3′-end (Bracht *et al.*, 2004; Cai *et al.*, 2004; Lee *et al.*, 2004a). The pri-miRNA folds back on itself to form a hairpin structure and then undergoes sequential nuclear and cytoplasmic processing to yield mature duplex miRNA.

The first step in the sequential processing of pri-miRNAs into mature miRNAs is promoted by the microprocessor comprising RNase III Drosha and its protein partner Pasha (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler *et al.*, 2004; Tomari and Zamore, 2005). Recognition of pri-miRNA by the microprocessor is facilitated by the presence of three RNA elements of the primary miRNA transcript: ≈80-nucleotide (nt) hairpin, unstructured ≥10-nt terminal loop, and single-single stranded RNA extensions beyond the pri-miRNA hairpin (Fig. 4.2) (Lee *et al.*, 2003; Zeng and Cullen, 2003; Zeng and Cullen, 2005; Zeng *et al.*, 2005). Upon recognition of pri-miRNA, Pasha determines the cleavage site at 11 base pairs away from the basal junction between the single-stranded RNA and double-stranded RNA of the pri-miRNA and ≈22 bp away from the apical junction of the terminal loop. Drosha cleaves the pri-miRNA at the Pasha-determined sites to release the 50–70 nt hairpin-structured pre-miRNA with two nucleotide 3′ overhang (Lee *et al.*, 2002; Gregory *et al.*, 2004; Han *et al.*, 2004). Following nuclear processing by the microprocessor, the pre-miRNA is then shuttled out of the nucleus by the nuclear transport factor Exportin-5 (Exp-5) in complex with Ran-GTP (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004).

In the cytoplasm, the pre-miRNA is further processed by the RNase III enzyme Dicer-1 (Dcr1) and its protein partner Loquacious (in flies). Dcr1 is a highly conserved protein that typically bears PAZ and two RNase III domains. The PAZ domain of *D. melanogaster* Dcr1 (Lee *et al.*, 2004b) recognizes the 2-nt 3′ overhang at the base of the pre-miRNA stem, and each of the 2 RNase domain then cleaves one of the strands off the loop to liberate the ≈22-nt-long miRNA duplex with each strand bearing 2-nt overhangs at 3′-end (Lee *et al.*, 2002; MacRae *et al.*, 2007).

Non-canonical biogenesis of host miRNA

Some miRNAs, termed non-canonical miRNAs, bypass one or more of the catalytic steps in the canonical miRNA biogenesis (Xie and Steitz, 2014). Non-canonical miRNAs are produced from introns and various RNAs such as small nucleolar RNAs (snoRNAs), endogenous short hairpin RNAs (shRNAs), and tRNAs (Abdelfattah *et al.*, 2014). The most prominent non-canonical maturation pathway, the mirtron pathway, substitutes Drosha cleavage with splicing. Splicing, carried out by spliceosomes and debranching enzymes, produces miRNA hairpins directly suitable for Dicer cleavage. After splicing, the mirtron pathway then merges with the canonical miRNA pathway at the Exp-5-bound transport stage (Okamura *et al.*, 2007).

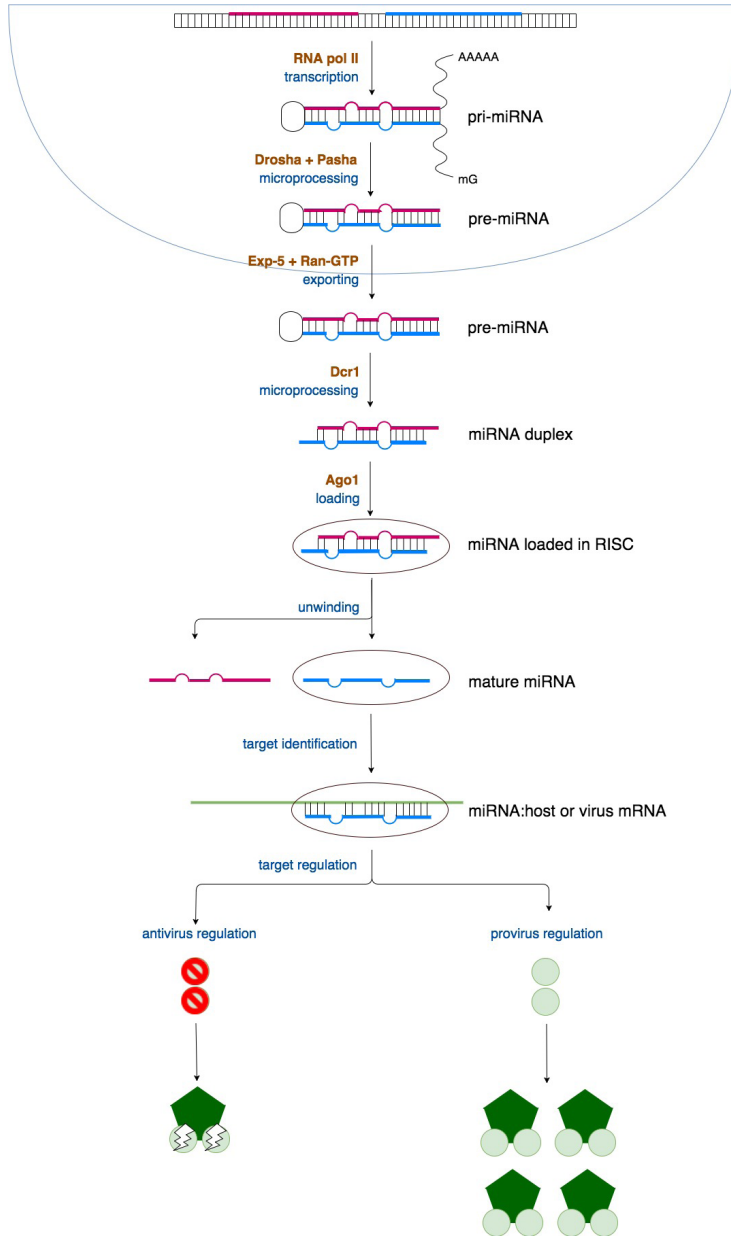

Figure 4.1 From canonical miRNA biogenesis to regulation of virus replication. The canonical biogenesis of miRNA begins with RNA polymerase II (RNA pol II)-mediated transcription of a miRNA gene. The resulting hairpin-containing primary transcript, pri-miRNA, spontaneously folds into a hairpin structure and is methylguanosine-capped at the 5′-end and may be polyadenylated at the 3′-tail. The pri-miRNA is then cleaved by Drosha, in association with Pasha, to generate the pre-miRNA. The pre-miRNA is shuttled out of the nucleus by the nuclear transport factor Exportin-5 (Exp-5), in association with Ran-GTP, for further processing by the cytoplasm-located Dicer-1 (Dcr1) and its partner Loquacious. Dcr1 cleaves the hairpin loop off the pre-miRNA to generate a ≈22 nt long miRNA duplex. The miRNA duplex is then loaded onto the effector molecule Argonaute (Ago) to form the RNA induced silencing complex (RISC). In the RISC, one strand of the miRNA duplex is unwound and discarded, while the other is retained to guide the effector complex to target mRNA. miRNA target recognition occurs through perfect or near perfect complementary binding between miRNA seed region (second to eighth nucleotides from the 5′ end of the miRNA) and the target mRNA. In the host–virus interaction, the target can be either host mRNA or virus mRNA. Upon binding, target mRNA stability and/or translational efficiency is modified leading to regulation of gene expression of virus or host factors which consequently impacts virus replication and/or pathogenesis. Where target regulation is provirus, virus replication is enhanced, conversely, where target regulation is antivirus, virus replication is restricted.

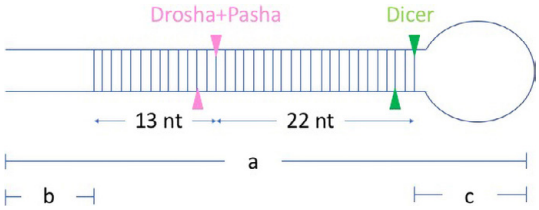

Figure 4.2 RNA elements in pri-miRNA required for Drosha recognition. (a) ≈80-nt long hairpin, (b) single-stranded extension beyond pri-miRNA hairpin, and (c) ≥10 nt terminal loop. (Modified from Bartel, 2018.)

The classical definition of miRNAs anchors heavily on the size of the RNA molecule and the biogenesis. Specifically, (1) the mature miRNA must have the distinct size of ≈22 nt, (2) the mature miRNA should occupy the stem part of a hairpin-shaped precursor that does not have large internal bulges, and (3) the mature miRNA should be processed by Dicer (Berezikov *et al.*, 2011). However, there exists a non-canonical miRNA pathway that bypasses Dicer cleavage. Mature miR-451 in humans, mouse, and zebrafish are produced by Drosha-cleavage of pri-miR-451 to generate a pre-miRNA that is too short (≈18 bp) to act as substrate for Dicer (Yang *et al.*, 2010). Whether there are other miRNAs produced through this unique miRNA biogenesis pathway is still unknown, and why seemingly only miR-451 is produced this way remains one of the larger unresolved miRNA questions (Bartel, 2018).

Biogenesis of virus miRNA

DNA viruses can encode miRNAs in their genome, the biogenesis of which parallels the biogenesis of host miRNAs. DNA virus miRNAs can be derived from non-coding RNAs and open reading frames of protein-coding mRNAs. In several cases, viral miRNAs are expressed from polycistronic transcripts, thereby ensuring their co-regulation. Similar to host miRNAs, most known DNA virus miRNAs are derived from Pol II transcripts. However, some miRNAs produced by viruses such as murine gammaherpesvirus 68 (MHV68), *Bovine leukaemia virus* (BLV), and *Simian foamy virus* (SFV), appear to be derived from transcripts produced by RNA polymerase III (Pol III). Most virus pri-miRNA transcripts then undergo Drosha and then Dicer cleavage. However, as with host miRNAs, some virus miRNAs are produced via the non-canonical pathway which bypasses the Drosha cleavage (Tycowski *et al.*, 2015).

Most known virus miRNAs are encoded by DNA viruses, although miRNAs/miRNA-like sequences can also be derived from RNA virus genome. While the biogenesis of RNA virus miRNAs has yet to be defined, the production of functional miRNAs from a pre-miRNA sequence engineered into the Influenza virus (nuclear RNA virus) genome suggests that RNA viruses are capable of producing miRNAs. Furthermore, the production of miRNAs capable of conferring RNAi-like activity from pre-miRNAs cloned into *Sindbis virus* (cytoplasmic RNA virus), called virtrons, suggests the existence of an uncharacterised Exp-5-independent, Dcr-dependent pathway capable of processing cytoplasmic hairpins (Shapiro *et al.*, 2010; Varble *et al.*, 2010; Langlois *et al.*, 2012; Asgari, 2015).

RISC assembly and miRNA strand selection

Whether miRNAs mature through the canonical or non-canonical pathway, RNAi-induced silencing complex (RISC) assembly follows the biogenesis of the miRNA duplex (Fig. 4.1). During RISC formation the miRNA duplex is loaded onto an Argonaute (Ago) protein. Ago proteins are highly conserved and specialized ≈100 kDa small-RNA-binding modules containing PAZ and PIWI domains. While some organisms such as the fission yeast, *Schizosaccharomyces pombe*, express only one type of Ago, others express multiple Ago family members. The model insect *D. melanogaster* expresses five Ago proteins (Hock and Meister, 2008). In *D. melanogaster*, the miRNA duplex, which typically contains central mismatches, associates with Ago1. Provided the miRNA duplex has no central mismatches, the miRNA is loaded onto the *D. melanogaster* Ago typically used for siRNAs, Ago2 (Okamura *et al.*, 2004; Förstemann *et al.*, 2007; Tomari *et al.*, 2007; Czech *et al.*, 2009; Ghildiyal *et al.*, 2010). Following loading onto the Ago protein, the strand with the more stable base pairs at the 5′-end, excess pyrimidines, and C at nucleotide position 1 is typically removed. The strand that normally gets removed has traditionally been termed miRNA star strand, miRNA*, or miR* or passenger strand (see Box 4.1 for naming

Box 4.1 What's in a mature miRNA name?

Except for the miRNAs bantam, let-7, and lin-4, a typical mature miRNA name follows the xxx-miR-yy-zp convention, where the first x represents the first letter of organism's genus name, and the last two xx represent the first 2 letters of the organism's species name. y indicates the sequential discovery of the specific miRNA. zp may be 5p or 3p, where 5p means that the mature miRNA was derived from the 5′ arm of the pre-miRNA, and 3p means that the mature miRNA came from the 3′ arm of the pre-miRNA (Fig. 4.1).

Mature miRNA names were previously formatted as xxx-miR-yy or xxx-miR-yy*, where the former represents the more abundant strand, and the latter the less abundant strand (miR*). There is generally a bias towards one strand of the miRNA duplex in the mature miRNA pool. This bias stems from the increased miRNA half-life and stability imparted by guide miRNA association with Ago (Winter and Diederichs, 2011), whereby Ago1 structure may shield the 5′ and 3′-ends of miRNA from ribonuclease action, thus protecting it from degradation (Wang *et al*., 2008). The contrast in the abundance of the two strands of miRNAs led to the misconception that while one arm is used for RNA-induced silencing, the other arm is simply degraded. However, improvements in miRNA profiling technologies allowed for a fraction of the traditionally termed miR* to be easily detected (Okamura *et al*., 2008). The ratio of each of the strands relative to the other is now known to vary depending on the tissue, developmental stage, and physiological state of the organism. The recognition that either or both strands of the miRNA duplex can serve as guide miRNA depending on the biological state (Okamura *et al*., 2008; Yang *et al*., 2011) prompted the shift in miRNA nomenclature from the traditional miR/miR* to miR-3p and miR-5p (Desvignes *et al*., 2015).

conventions). The strand with the lesser inter-strand hydrogen bonding resistance, excess purines, and U at nucleotide position 1 is normally retained. This strand becomes the guide miRNA strand, also known as the miRNA, or miR (Khvorova *et al*., 2003; Czech *et al*., 2009; Hu *et al*., 2009; Okamura *et al*., 2009; Ghildiyal *et al*., 2010).

miRNA recognition of targets

With the miRNA strand specified and selected, the miRNA is now ready to guide the RISC complex to recognize mRNA transcripts. Ago interacts with the miRNA phosphate backbone to splay out the seed sequences in a helical conformation, exposing the bases for target binding (Ma *et al*., 2004; Nakanishi *et al*., 2012; Schirle and MacRae, 2012; Schirle *et al*., 2014). miRNA targets are recognized via Watson–Crick base pairing between the miRNA and the mRNA. While there are many types of miRNA–mRNA pairs, the most common type involves miRNA nucleotides 2–8 from the 5′-end (termed miRNA seed) binding with mRNA (Lewis *et al*., 2003; Brennecke *et al*., 2005; Krek *et al*., 2005; Lewis *et al*., 2005; Bartel, 2009) (Fig. 4.3). The importance of miRNA seed region is associated

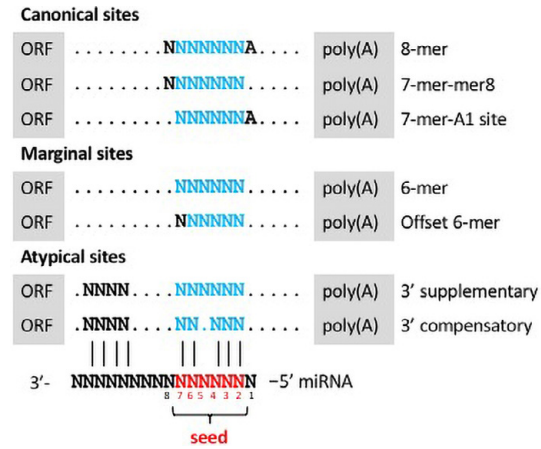

Figure 4.3 miRNA seed types. (Modified from Bartel, 2009.)

with the mechanistic effect of miR seed–target pairing on RISC association. Specifically, during Ago movement along the mRNA target, base pairing of miRNA nucleotides 2–5 contribute to primary RISC binding, while nucleotides 6–8 slow down RISC dissociation after encountering the target. This reduction in the rate of RISC dissociation

strengthens the interaction between the miRNA, target, and RISC, resulting in increased efficacy of RISC (Brennecke *et al.*, 2005; Grimson *et al.*, 2007).

miRNA regulation of target

Following mRNA target recognition, miRNA facilitated target regulation occurs. Repression of gene expression is the most common mode of regulation. In the rare case of full complementarity between the miRNA and target, the mRNA is endonucleolitically cleaved by Ago, as is common in plants. In animals, however, the complementarity is most often imperfect, and target repression occurs through mRNA decay and/or mRNA translational repression. mRNA decay has been shown to account for 66–90% of the miRNA-mediated repression, thus contributing substantially to overall silencing (Jonas and Izaurralde, 2015). mRNA decay involves mRNA deadenylation, followed by decapping, and final (in the current model) degradation by cytoplasmic nuclease 5′-to-3′ exoribonuclease (Guo *et al.*, 2010; Huntzinger and Izaurralde, 2011; Djuranovic *et al.*, 2012; Wilczynska and Bushell, 2015; Jonas and Izaurralde, 2015). Mounting evidence suggests that translational repression in association with a 182 kDa phosphoprotein bearing glycine-tryptophan repeats (GW182) precedes mRNA decay (Baek *et al.*, 2008; Selbach *et al.*, 2008; Ingolia *et al.*, 2009; Huntzinger and Izaurralde, 2011; Bazzini *et al.*, 2012; Djuranovic *et al.*, 2012; Wilczynska and Bushell, 2015), however the mechanism(s) through which miRNAs repress translation of mRNAs is still unclear (Iwakawa and Tomari, 2015). Current models propose several concurrent and overlapping mechanisms that could account for translational repression, including: GW182-mediated recruitment of translational repressors and GW182-mediated displacement of poly(A)-binding proteins (PABP), which subsequently interferes with the mRNA circularization and therefore translation (Eulalio *et al.*, 2009; Lian *et al.*, 2009).

While most examples of miRNA-mediated regulation show target repression, miRNAs can also stimulate gene expression (Vasudevan *et al.*, 2007; Ørom *et al.*, 2008; Bruno *et al.*, 2011; Hussain *et al.*, 2011; Vasudevan, 2012). Positive regulation of genes by miRNAs can occur as a result of miRNA targeting RNA decay machineries (Bruno *et al.*, 2011) or as a result of miRNA associations that decrease levels of GW182 (Vasudevan, 2012).

miRNA roles in host–virus interaction

miRNA-mediated differential gene expression is a cornerstone event of biological processes. Each miRNA can potentially target hundreds of mRNAs (Enright *et al.*, 2004; Farh *et al.*, 2005; Krek *et al.*, 2005; Stark *et al.*, 2005; Sood *et al.*, 2006; Baek *et al.*, 2008; Betel *et al.*, 2010; Hashimoto *et al.*, 2013), and miRNA-mediated changes in gene expression have been implicated in a wide variety of functions. In insects, miRNAs have been implicated in growth, muscle and wing development, neurogenesis, apoptosis, sex determination, phenotypic plasticity, oogenesis and embryogenesis, and host–pathogen interactions and immunity (Asgari, 2013; Lucas *et al.*, 2015)

Virus infection changes the host miRNA profile such that the miRNA abundance in uninfected and infected cells, tissues and organisms differ (Scaria *et al.*, 2006; Dykxhoorn, 2007; Pedersen *et al.*, 2007; Gottwein and Cullen, 2008; Lindsay, 2008; Lodish *et al.*, 2008; Tsitsiou and Lindsay, 2009; Xiao and Rajewsky, 2009; O'Connell *et al.*, 2010; Cullen, 2011; Libri *et al.*, 2013; Asgari, 2015; Trobaugh and Klimstra, 2017; Monsanto-Hearne and Johnson, 2018). Indeed, differential abundance of miRNAs during viral infection is often used to identify which miRNAs may function in host–virus interactions (Asgari, 2015; Monsanto-Hearne and Johnson, 2018). The role of the differentially abundant miRNAs is verified based on the impact of miRNA regulation on the host and the virus (Fig. 4.4). The impact of miRNA abundance changes during virus infection is assessed by manipulation of miRNA levels via loss-of-function (LOF) or gain-of-function (GOF) methods (Min and Yoon, 2010; Martinez-Sanchez and Murphy, 2013) often employing miRNA inhibitors and miRNA mimics, respectively. miRNA inhibitors are chemically synthesized single-stranded RNA molecules designed to specifically bind to, inhibit, and artificially down-regulate endogenous miRNAs, while miRNA mimics are chemically synthesized double-stranded RNAs that artificially simulate up-regulation of miRNAs by augmenting endogenous miRNAs (Kuhn *et al.*,

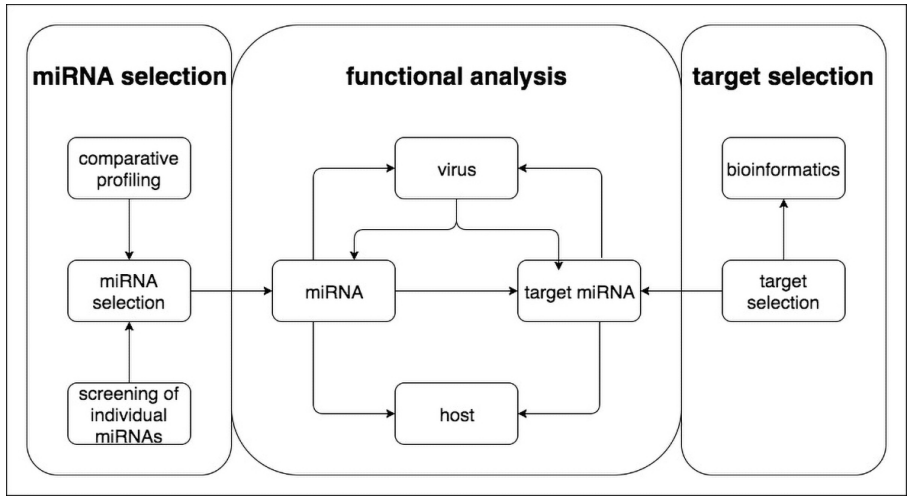

Figure 4.4 Pipeline and criteria for analysis of miRNA function in the host–virus interaction. Comparison of miRNA profiles of uninfected and virus-infected host identifies miRNAs which are differentially regulated during virus infection. Once a miRNA of interest has been identified (left panel), putative targets are identified using various bioinformatics approaches (right panel). Once miRNA–target interaction is confirmed, a closed-loop inter-relationship amongst the (1) miRNA, (2) target gene, (3) virus, and (4) host has to be confirmed (centre panel) so that the miRNA–target pair can be categorically identified as being relevant to the host–virus interaction. Where LOF/GOF mutants exist, identification of relevant miRNAs can start with genetic screens (left panel). (Modified from Monsanto-Hearne and Johnson, 2018.)

2008; Thomson *et al.*, 2011; Chugh and Dittmer, 2012; Ekimler and Sahin, 2014). Other common methods for LOF use miRNA sponges, tough decoys, target protectors, miRNA activity sensors, and target sensors (Chugh and Dittmer, 2012; Steinkraus *et al.*, 2016). Other methods for GOF use expression vectors containing mature miRNA, precursor miRNA, or pri-miRNA sequences (Min and Yoon, 2010; Thomson *et al.*, 2011). To test the effect of changes in miRNA abundance on host–virus interactions, miRNA inhibitors and mimics are introduced into cells by transfection, or to whole organisms *per os* or by injection. Involvement of a miRNA in host–virus interaction is evidenced by LOF and/or GOF resulting in changes in parameters such as cytopathic effects, host survival, and virus titres (Ekimler and Sahin, 2014) (see Box 4.2).

miRNAs exert their physiological effects through target mRNAs. Once a specific miRNA's change in abundance has been confirmed to affect host-virus replication, the next step in functional analysis typically involves identification of putative targets using bioinformatics. Several computational filtering prediction tools that generate a list of putative targets based on miRNA-target seed match and other experimentally defined features are now available. Different miRNA-target prediction algorithms generate different output lists with their associated false-positive and false-negative rates (Ritchie *et al.*, 2009; Thomson *et al.*, 2011). Identifying putative targets that are commonly identified by two or more algorithms is commonly employed to reduce the false-positive rates (Min and Yoon, 2010; Witkos *et al.*, 2011; Martinez-Sanchez and Murphy, 2013; Ekimler and Sahin, 2014; Riffo-Campos *et al.*, 2016). Putative targets are then experimentally validated using small-scale or genome-wide experimental approaches. In small-scale studies, RT-qPCR, *in situ* hybridization, northern blot, western blot, and protein arrays are used to measure changes in the target gene or protein after perturbation in miRNA levels. Because miRNAs potentially regulate hundreds of mRNA targets, large-scale transcriptional and proteomic profiling allows for a simultaneous and global assessment of targets following modulation of miRNA activity. Differences in target levels can then be quantitatively compared between control samples and LOF/GOF samples, and between control samples and virus-infected samples (Steinkraus *et al.*, 2016).

Box 4.2 Functional analysis *in vivo*

The use of cell cultures has substantially contributed to the functional analysis of miRNA roles in host–virus interaction. In particular, *ex vivo* approaches have been useful for documenting the physical interactions between miRNAs and their targets (Steinkraus *et al*., 2016). However, disrupting miRNA activity *in vivo* has allowed for spatially and temporally synchronized data, providing more evidence on the definitive biological functions of miRNAs (Monsanto-Hearne and Johnson, 2018).

The development of miRNA LOF and GOF in whole organisms already has some success stories. Delivery of inhibitors and mimics *per os* (Jayachandran *et al*., 2012) or by injection (Joo *et al*., 2007) have both successfully resulted in change in the magnitude of miRNA effects on targets. There is also now an extensive collection of *D. melanogaster* miRNA stocks with miRNA LOF and GOF. For LOF, knockout mutant flies and lines with transgenes that 'sponge' or sequester miRNAs have been produced. For GOF, lines that use the Gal4-UAS system for miRNA expression have been developed (Bejarano *et al*., 2012; Schertel *et al*., 2012).

The final step of miRNA functional analysis is the evaluation of the functional impact of the target mRNA in the host–virus interaction. This requires manipulation of the target mRNA levels by GOF or LOF. The use of RNAi to knock down the target gene is the most commonly used technique in insect miRNA functional studies. Confirmation that the identified target mRNA functions in host–virus interaction occurs when the knockdown of the target mRNA results in changes in determinants of pathogenesis.

Host miRNA roles in insect–virus interactions

miRNA profiling and functional analysis of insect host–virus interactions have shown that miRNA abundance changes in the host can influence the outcome of virus pathogenesis (Asgari, 2015). The most thoroughly investigated insect hosts in the context of virus infection are mosquitoes of the *Aedes* and *Culex* genera. The involvement of miRNAs during virus infection has also been explored in the silkworm *Bombyx mori*, the moths *Helicoverpa armigera*, *Heliothis virescens*, *Spodoptera frugiperda*, and *Spodoptera litura*, a bumble bee *Bombus terrestis*, a plant hopper *Laodelphax striatellus*, the cabbage looper *Trichoplusia ni*, and in vinegar flies *D. melanogaster*. Different insect hosts, tissue sample sources, viruses, methods of infection, and sampling times post-infection have been used in miRNA profiling studies. Despite this, common patterns from the literature on the insect–virus interaction have emerged: (1) some miRNAs are commonly altered by virus infection, (2) the number of differentially regulated miRNAs changes over the course of virus infection, (3) the direction of regulation of a miRNA can change depending on the time post-infection, and (4) the general direction of change in miRNA abundance can vary depending on tissue sample source. Further functional analyses of the differentially abundant miRNAs show that insect host miRNAs can target either host mRNA or virus mRNA and can either have antivirus or provirus consequences (Fig. 4.5) (Monsanto-Hearne and Johnson, 2018). Details on the host miRNAs that have been functionally characterized in insect–virus interactions are described below.

Host miRNA-host mRNA interaction with antivirus consequence

Host miRNAs are first and foremost for the host to use. Various insect miRNAs inhibit viral replication and protect the host from viral attack. Here we summarize the literature on the involvement of insect host miRNA in antiviral mechanisms.

Infection of the Asian tiger mosquito *Aedes albopictus* with the medically important positive-sense ssRNA flavivirus *West Nile virus* (WNV) is regulated by mosquito host miRNA aae-miR-2940-5p. Mosquito aae-miR-2940-5p is selectively down-regulated during virus infection of the *Ae. albopictus* C6/36 cell line. Artificial miRNA down-regulation using synthetic aae-miR-2940-5p inhibitors represses WNV replication (Fig. 4.6). Conversely, artificial miRNA up-regulation using synthetic aae-miR-2940-5p mimic facilitates increase in

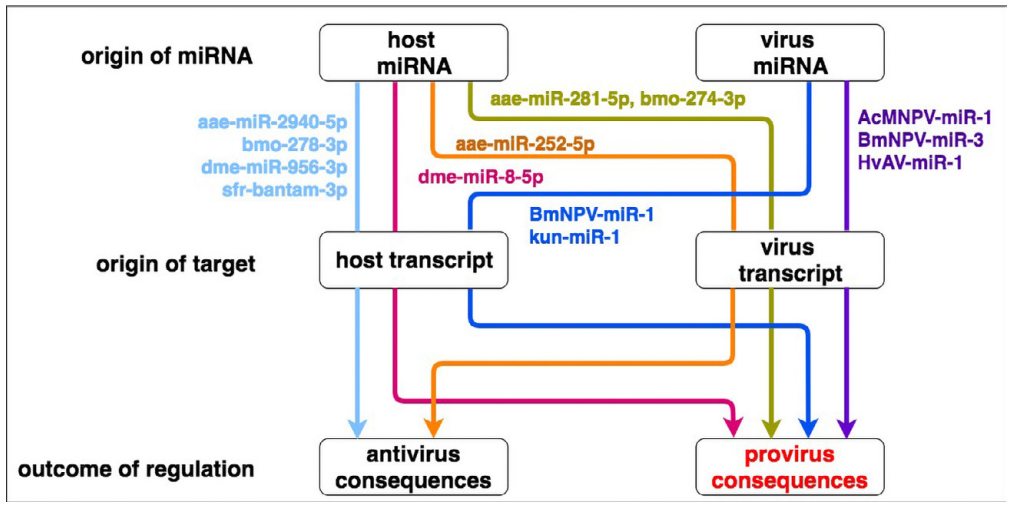

Figure 4.5 Both host and virus-derived miRNAs can target either host or virus mRNA transcripts. Host miRNA can target host or virus miRNAs and can either have pro-host or pro-virus consequences whereas virus-derived miRNAs can target either host or virus miRNAs but lead only to pro-virus consequences. miRNA with described functions are mapped to the pathway of impact.

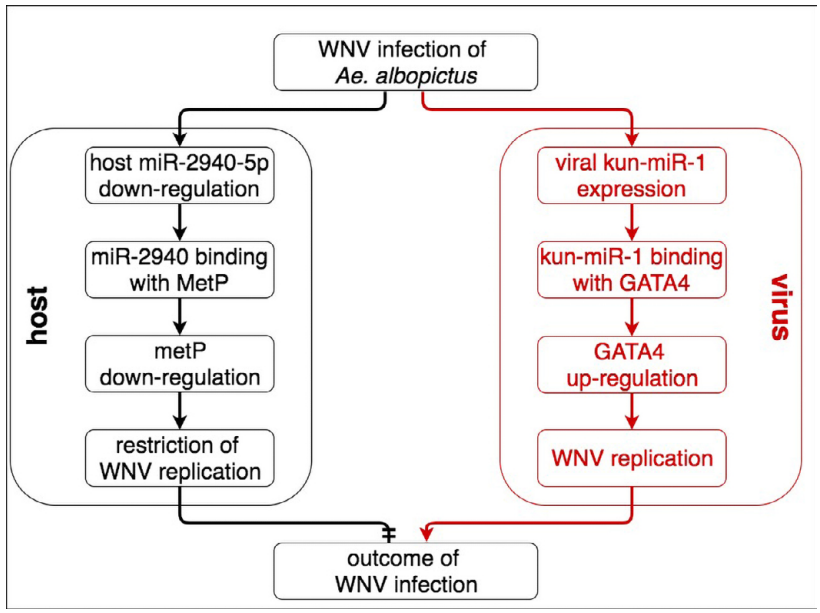

Figure 4.6 The contrasting effects of host miRNA and virus miRNA regulation of host mRNA in *Ae. albopictus*–WNV interaction. Black arrows antivirus pathway, in contrast, red arrows denote provirus pathway.

WNV titres, suggesting that the infection-induced miRNA down-regulation negatively impacts virus infection. Infection-induced decrease in aae-miR-2940-5p levels lowers the expression of *metalloprotease m41 filamentous temperature sensitive H* (*MetP m41 FtsH*). Experimental knockdown of *MetP* also negatively impacts virus infection. The cascade of events beginning with infection-induced down-regulation of miR-2940-5p ends with restriction of WNV infection. Although the molecular mechanism that leads to *MetP*'s negative impact on WNV replication is not yet understood, this

cascade of events shows that miR-2940-5p regulation during virus infection is a miRNA-dependent antiviral response (Slonchak *et al.*, 2014).

Bombyx mori cytoplasmic polyhedrosis virus (BmCPV) infection of the mulberry silkworm *B. mori* is regulated by host miRNA that binds to host miRNA. Oral infection of *B. mori* with the segmented dsRNA reovirus BmCPV by feeding newly exuviated fifth instar silkworm larvae down-regulates host bmo-miR-278-3p in the midgut (Fig. 4.7). The infection-induced decrease in miR-278-3p then leads to an increase in transcription of the *insulin-related peptide binding protein 2* (*IBP2*) gene. Conversely, experimental increase in miR-278-3p by injection of miR-278-3p mimic into silkworm larvae decreases *IBP2* transcripts. An increase in miR-278-3p in turn increases the mRNA transcript of virus polyhedrin, which is a proxy for measuring BmCPV replication. Although a direct relationship between IBP2 levels and BmCPV has not yet been established, it is hypothesized that IBP2 plays a role in the insulin-signal pathway, restricting BmCPV replication as part of a silkworm immune response (Wu *et al.*, 2016).

The infection of *S. litura* larvae and Sf9 cells with the baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) increases the abundance of the miRNA bantam. The up-regulation of bantam using an miRNA mimic is associated with a dose-dependent decrease in viral accumulation in cells. In contrast, artificial down-regulation of bantam using a synthetic inhibitor in cells increases viral accumulation in a dose dependent manner. In addition, *per os* delivery of the miRNA bantam antagomir into *S. litura* from the second instar larvae to pre-pupal stages results in accelerated mortality. A number of genes were concomitantly regulated with bantam abundance change during infection, so may also be involved. The detailed nature of the interaction between bantam and the mRNAs requires further experimentation. Additionally, the relationship between the mRNAs and the virus has yet to be tested (Shi *et al.*, 2016).

Injection of the model organism *D. melanogaster* with its natural pathogen *Drosophila C virus* (DCV) down-regulates dme-miR-956-3p (Monsanto-Hearne *et al.*, 2017b). The decrease in miR-956-3p

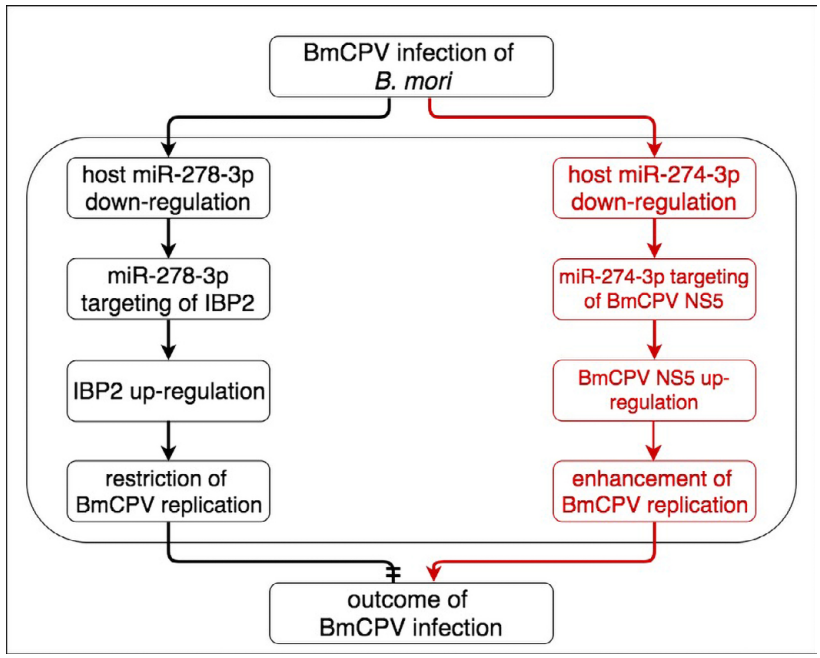

Figure 4.7 The contrasting effects of host miRNA regulation of host mRNA and host miRNA regulation of virus miRNA in *B. mori*–BmCPV interaction. Black arrows antivirus pathway, in contrast, red arrows denote provirus pathway.

abundance during DCV infection up-regulates the expression of the gene *Ectoderm-expressed 4* (*Ect4*), as shown by the higher levels of *Ect4* in mutant flies with a loss-of-function deletion of miR-956 when compared to *Ect4* in wild-type flies with normal levels of miR-956. This DCV-induced miR-956-3p down-regulation, which consequently up-regulates *Ect4*, correlates with slower viral accumulation and slower fly mortality, thus demonstrating that miRNA-mediated host protection is exerted through changes in host gene expression (Fig. 4.8).

Host miRNAs–virus mRNA interaction with antivirus consequences

Host miRNA can also protect hosts against virus infection through host miRNA interaction with virus mRNA. Infection of *Ae. albopictus* C6/36 cells with the flavivirus *Dengue virus* serotype 2 (DENV-2) induces miR-252-5p. Artificial up-regulation of miR-252-5p in cultured cells by means of transfection of synthetic oligonucleotides mimicking miR-252-5p decreases DENV-2 E protein RNA and protein, and therefore DENV-2 replication. Conversely, artificial down-regulation of miR-252 using inhibitors increases DENV-2 E protein RNA and protein, demonstrating an inverse relationship between miR-252 and DENV-2 E protein. This suggests that miR-252-3p up-regulation acts as a repressor of DENV-2 E protein, and consequently of DENV-2 replication (Fig. 4.9) (Yan *et al.*, 2014). It is noteworthy that, although acutely infected C6/36 cells have up-regulated levels of miR-252-5p, persistently infected C6/36 cells have down-regulated miR-252-5p (Avila-Bonilla *et al.*, 2017). It will be interesting to explore the implications of miR-252-5p down-regulation of DENV-2 persistent infection of cells.

Host miRNAs–host mRNA interaction with provirus consequences

Functional analyses of miRNAs during virus infection show that differential abundance of host miRNAs could result from viral manipulation of the host machinery required for virus proliferation. Provirus effects of miRNAs can occur through interaction between host miRNA and cellular mRNA. During DCV infection of *D. melanogaster* adult flies, miR-8-5p is down-regulated (Monsanto-Hearne *et al.*, 2017a). The decrease in miR-8-5p abundance during DCV infection up-regulates the expression of *Jun-related antigen* (*Jra*), also known as *dJun* due to its homology with human *Jun*. The

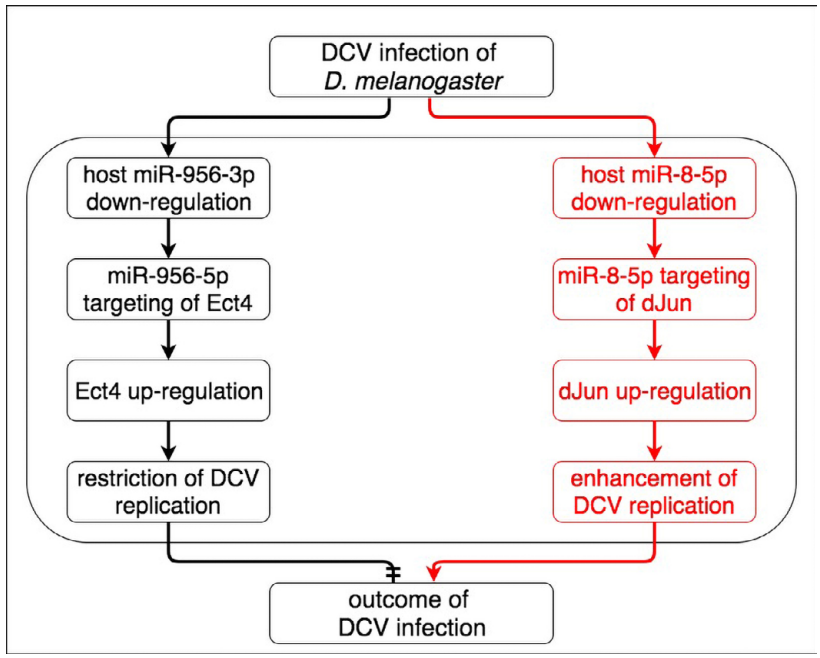

Figure 4.8 The contrasting effects of host miRNA regulation of host mRNA in *D. melanogaster*–DCV interaction. Black arrows antivirus pathway, in contrast, red arrows denote provirus pathway.

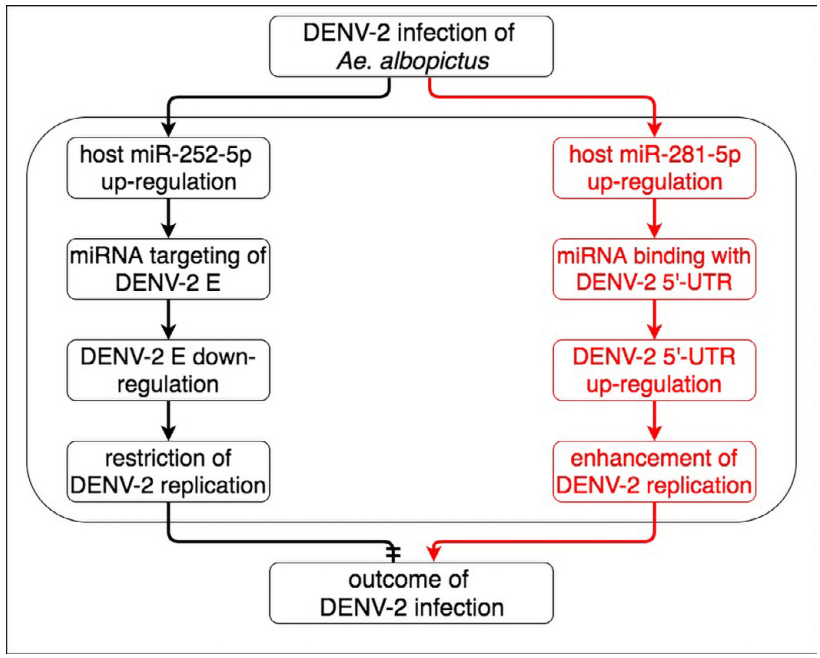

Figure 4.9 The contrasting effects of host miRNA regulation of virus mRNA in *Ae. albopictus*–DENV interaction. Black arrows antivirus pathway, in contrast, red arrows denote provirus pathway.

increase in *dJun* then results in faster viral accumulation. *dJun* expression is widely known to be regulated by protein phosphorylation, which then positively auto-regulates its transcription to create more *dJun* (Smeal *et al.*, 1994). *dJun* up-regulation resulting from miR-8-5p down-regulation shows that the miRNA pathway contributes to *dJun* induction during viral infection (Fig. 4.8). Thus, there is complementarity of different regulatory networks determining the gene expression of host factors during viral infection. This demonstrates the ability of viruses to subvert multiple host regulatory pathways to enable viral replication.

Host miRNA–virus mRNA interaction with provirus consequences

Provirus effects of miRNAs can also occur through interaction between host miRNA and virus mRNA. Infection of silkworm with BmCPV is not only regulated by silkworm miRNA targeting host mRNA (see above), but also by host miRNA targeting virus mRNA. Oral infection of silkworm with BmCPV down-regulates miR-274-3p, which up-regulates the non-structural protein RNA of the BmCPV genome segment NS5, as shown by a direct negative interaction between the host miRNA and the virus NS5 segment in cells (Wu *et al.*, 2017). Although NS5 segment RNA was down-regulated by miR-274-3p mimic, the transcript was unaffected by negative mimic control. Additionally, microinjection of miR-274-3p inhibitors into silkworm larvae enhanced NS5 mRNA and protein expression. Importantly, artificial down-regulation of miR-274-3p also increased virus polyhedrin expression, which was used as proxy for virus replication. This suggests that infection-induced miR-274-3p inhibition enhances BmCPV replication. This contrasts with bmo-miR-278-3p, which acts by increasing the abundance of the *IBP2* gene, which is hypothesized to then inhibit BmCPV replication (Fig. 4.7).

The provirus effect of host miRNA regulation of virus mRNA also occurs in the *Ae. albopictus*–DENV-2 interaction. During oral DENV-2 infection of *Ae. albopictus*, miR-281-5p is up-regulated in the mosquito midgut, with miR-281-5p positively interacting with DENV-2 genomic 5′-untranslated region (UTR) to facilitate DENV-2 replication (Fig. 4.9). The artificial up-regulation of miR-281-5p prior to DENV-2 infection increases DENV-2 genomic RNA (gRNA), DENV-2 E protein, and therefore DENV-2 replication in C6/36 cells (Zhou *et al.*, 2014). In contrast, artificial

down-regulation of miR-281-5p prior to infection reduces viral gDNA levels, DENV-2E protein, and DENV-2 titres in cells. Knock-down of miR-281-5p in mosquitoes *in vivo* through antagomir-281 injection also reduces viral gDNA, demonstrating that the miR-281-5p impact on DENV-2 occurs in both cells and whole organisms.

Host miRNA–virus mRNA interaction with unknown consequences

During early infection of a *Heliothis virescens* fat body cell line with *Heliothis virescens ascovirus 3a* (HvAV-3a), *H. virescens* miRNA Hz-miR-24 is down-regulated in cells; however, the same miRNA is up-regulated in late infection (Hussain and Asgari, 2010). During late infection, the transcript levels of DNA-dependent RNA polymerase (DdRP, *ORF64*) and DdRPβ (*ORF82*) are decreased, corresponding to higher levels of miR-24-5p. Artificial increase of miR-24-5p by cloning the pre-miRNA sequence of miR-24-5p and ectopically expressing the miRNA decreased the transcript levels of DdRP and DdRPβ compared to the levels in control transfection. Conversely, artificial decrease of miR-24-5p by synthetic miR-24-5p inhibitor increased DdRP expression. Neither artificial miR-24-5p regulation nor silencing of DdRP and DdRPβ impacted virus replication, suggesting that miR-24-5p and its two identified targets are not essential for *in vitro* HvAV-3 replication. It has been hypothesized that the target genes may be important for the expression of late genes and production of virions *in vivo*, but this requires testing (Hussain and Asgari, 2010).

Virus miRNA roles in host–virus interaction

In contrast to host miRNAs, which can either be antivirus or provirus, all virus miRNAs thus far discovered, regardless of whether the target is virus mRNA or host mRNA, are provirus elements. The provirus effects of virus miRNAs occur either through direct regulation of viral products or through directly regulating host transcripts (Aguado and tenOever, 2018).

Virus miRNA–virus mRNA interaction

Virus miRNA can bind to the virus mRNAs to exert provirus functions. This can result in enhanced virus replication. Alternatively, virus miRNA–virus mRNA interaction can regulate virus replication by facilitating transitions from one viral replication stage to another or by preventing early host recognition and immune response. Virus miRNA–virus mRNA binding could also potentially prevent the rapid death of the host, providing the virus with a viable host/virus reservoir for a longer period of time.

HvAV-3 produces HvAV-miR-1 from the 3′ stem-loop (3′SL) in the ORF coding for capsid protein at 96 hours post-infection. At the same time point, the viral DNA pol I (*orf1*) transcript is sharply down-regulated as a result of experimentally validated, direct binding of HvAV-miR-1 and the *orf1* transcript. Ectopic expression of HvAV-miR-1 in cells decreases viral DNA levels, suggesting that HvAV-miR-1 mediates inhibition of viral replication by specifically binding with *orf1* transcript. It is hypothesized that virus HvAV-miR-1 down-regulation of its own gene and its own replication delays host death, therefore conferring an advantage to long-term virus propagation (Hussain *et al.*, 2008).

The baculovirus AcMNPV also produces a miRNA, AcMNPV-miR-1. First identified bioinformatically, AcMNPV-miR-1 production was later experimentally validated. The experimentally validated miRNA perfectly matches and directly negatively targets a segment in the viral gene ODV-E25, reducing the level of infectious budded virions (Zhu *et al.*, 2013).

Finally, *Bombyx mori nucleopolyhedrovirus* (BmNPV) produces BmNPV-miR-3 during the early stage of infection of silkworm larvae. BmNPV-miR-3 negatively affects P6.9 expression and restricts viral replication such that P6.9 levels and viral replication decrease upon synthetic up-regulation of BmNPV-3. In contrast, P6.9 levels and virus replication increase upon synthetic down-regulation of the miRNA (Fig. 4.10). The control of P6.9 (and other late genes) by virus miRNA helps the virus escape early detection by the host, and therefore early immune response attack (Singh *et al.*, 2014).

Virus miRNA–host mRNA interaction

While some virus miRNAs bind to viral mRNA to exert provirus functions, other virus miRNAs achieve their provirus functions by regulating host

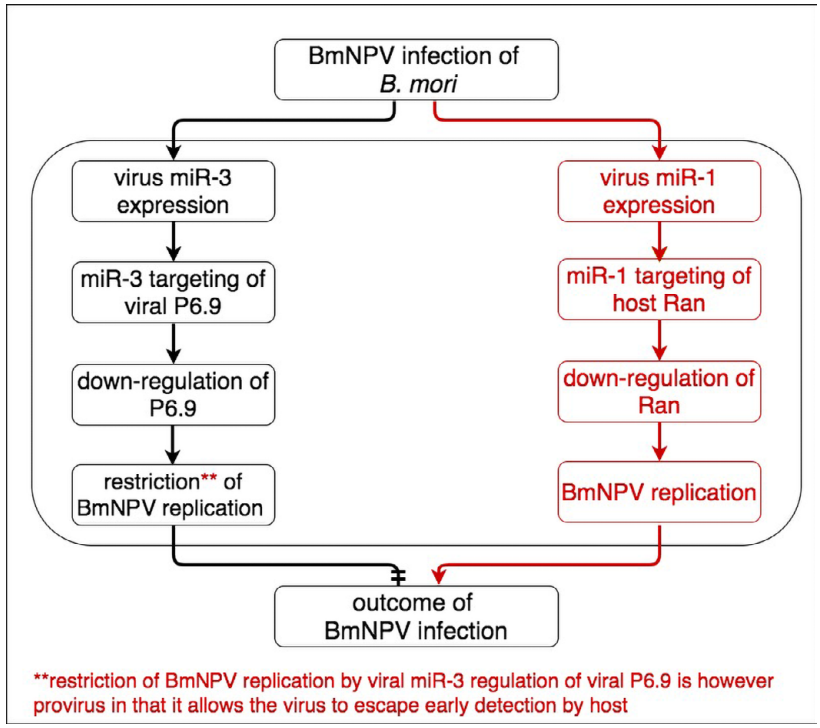

Figure 4.10 The contrasting effects of virus miRNA regulation of virus mRNA and virus miRNA regulation of host mRNA in *B. mori*–BmNPV interaction. Black arrows antivirus pathway, in contrast, red arrows denote provirus pathway.

gene expression. WNV encodes viral miRNA-like kun-miR-1 in WNV 3′SL. Using bioinformatics and cloning approaches, host *GATA4* was identified and confirmed as a kun-miR-1 target. Kun-miR-1 and *GATA4* interaction specifically up-regulates *GATA4*. Both the artificial decrease in kun-miR-1 levels by use of inhibitor and knockdown of *GATA4* by RNAi decreased WNV short flavivirus RNA (sfRNA), demonstrating that kun-miR-1 expression up-regulates *GATA4*, which then enhances WNV infection (Fig. 4.6) (Hussain *et al.*, 2012).

Virus miRNA–host miRNA biogenesis pathway interaction

Virus infection can lead to changes in the global miRNA profile through regulation of miRNA biogenesis. The infection of silkworms with BmNPV involves targeting of a host gene by baculovirus miRNA BmNPV-miR-1 (Fig. 4.10). BmNPV-miR-1 specifically targets the host gene *Ran*, which is involved in the export of pre-miRNAs from the nucleus to the cytoplasm during miRNA biogenesis (Singh *et al.*, 2010). The resulting impairment of miRNA biogenesis following BmNPV-miR-1:*Ran* interaction thus increases virus proliferation. This mechanism employed by BmNPV is particularly interesting because it changes the miRNA biogenesis itself. miRNA level changes through alteration of components of the miRNA biogenesis pathway is also seen during infection of *Bombus terrestris* with the avirulent *Slow bee paralysis virus* (SBPV). In this system, SBPV infection increases expression of Dcr1 and Ago1, demonstrating the importance of miRNAs in host–virus interaction (Niu *et al.*, 2017). Furthermore, it raises the possibility that down-regulation of miRNAs during DCV infection of *D. melanogaster* could be due to the impact of DCV-infection on the miRNA biogenesis pathway (Monsanto-Hearne *et al.*, 2017b).

Future directions

Current data on functional analysis of miRNAs during insect–virus interaction show that miRNAs are important determinants of host–virus interactions. Detailed knowledge of miRNAs in this

context may lead to important future applications. Examples include: interventions to address virus-infection of economically important insects such as silkworm and bees; as the basis for design of interventions for vector-borne diseases in humans, livestock, and crops; and finally, particularly for the insect model *D. melanogaster*, as the potential basis for understanding host–virus interaction in humans.

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